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Modification of isolated subunit c of the F_1F_o-ATPase from *Propionigenium modestum* by dicyclohexylcarbodiimide

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Abstract

Subunit c of the F_1F_o-ATPase from *Propionigenium modestum* was extracted from the particulate cell fraction with chloroform/methanol. The protein was further purified by carboxymethyl cellulose chromatography and anion exchange HPLC in the organic solvent. SDS-PAGE of the purified protein indicated a single stained protein band migrating as expected for the c-subunit. Incubation of isolated subunit c in chlorform/methanol or aqueous buffer containing dodecyl-β-D-maltoside with [^{14}C]dicyclohexylcarbodiimide (DCCD) resulted in the incorporation of radioactivity into the protein. The rate of this reaction depended on the external pH; it was significantly faster in the more acidic than in the alkaline pH range. In the presence of Na^+ subunit c was partially protected from labeling with [^{14}C]DCCD at pH 6.1 and at pH 7.5, whereas no protection was evident at pH 5.5. At pH 7.5, the rate of subunit c labeling by [^{14}C]DCCD in the presence of 20 mM NaCl was about 50% lower than in the absence of Na^+ ions. The isolated c-subunit therefore apparently retains in part the Na^+ binding site which, when occupied, diminishes the reactivity of the protein towards DCCD.

Key words: Na^+ binding site; Ion translocation; Na^+ pump

1. Introduction

ATP synthesis in the strictly anaerobic bacterium is entirely dependent on a unique type of F_1F_o-ATPase that uses Na^+ instead of H^+ as the physiological coupling ion [1]. Previous results indicated that the Na^+ binding site is located on the F_o part of the *P. modestum* ATPase [2,3]. In detailed studies on the mode of ion translocation across F_o we found that the mechanism is related to transporters rather than to channels [4]. More recently, evidence has been presented from the kinetics of ATPase inactivation by DCCD in relationship to Na^+ and H^+ concentration that the Na^+-binding site is at the DCCD-reactive highly conserved acidic residue in the middle of the C-terminal membrane-spanning α-helix of subunit c (Glu-65 in case of the *P. modestum* enzyme) [5]. Mutagenesis studies have indicated that the corresponding acidic residue of the *E. coli* enzyme (Asp-61) is of key importance for proton translocation (see [6] for a review). But whether this proton translocation involves protonation and deprotonation of Asp-61 by corresponding p*K* changes during the catalytic cycle [6] or binding and release of a hydronium ion by an ion-exchange type of mechanism [5] remains to be established. Of considerable importance in this context is the elucidation of part of the structure of the *E. coli* c-subunit by NMR methods, indicating a hairpin-like molecule with two hydrophobic membrane-spanning α-helical segments and a more hydrophilic connecting loop [7].

While our above mentioned results strongly indicate that the Na^+ binding site of the *P. modestum* ATPase is at Glu-65 of the c-subunit, it was not known whether other subunits contributed ligands for Na^+ binding or whether subunit c was sufficient to bind the metal ion. Here we report the labeling of isolated c-subunits by DCCD and partial protection from labeling by Na^+ in a pH-dependent manner. These results confirm and extend our previous conclusion that the Na^+ binding site is at Glu-65 of subunit c.

2. Experimental

2.1. Purification of subunit c of the ATPase (proteolipid) from P. modestum

The c-subunit was isolated and concentrated essentially as described [8]. The crude c-subunit was either purified by chromatography on CM cellulose (method A) [9] or by an anion-exchange HPLC column (method B). Briefly, 30 g *P. modestum* cells grown as described [1] were disrupted by passage through a French pressure cell in 120 ml buffer containing 50 mM potassium phosphate, pH 8.0, 1 mM dithioerythritol, and 0.1 mM diisofluorophosphate. After ultracentrifugation (1 h, 150,000 × *g*), membranes and cell debris were suspended in 20 volumes (v/w) of chloroform/methanol (2:1; v/v), and stirred overnight at 4°C. The suspension was then filtered through a fluted filter paper (No.

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; EIPA, *N*-ethyl-*N*-isopropylamiloride

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595½, Schleicher and Schuell AG, Switzerland) and 0.2 volumes of water were added. The lower phase and the interphase were collected and washed twice with chloroform/methanol/water (3:47:48). The solvent of the chloroform phase was almost completely removed by rotoevaporation at 30°C using the vacuum from a water-aspirator. The contents of the flask were taken up in 2.5 ml of chloroform/methanol (2:1). The c-subunit was subsequently precipitated by slowly adding four volumes of diethylether while gently shaking. After 20 h at 4°C, the precipitate was collected by centrifugation at 6,000 × *g* for 10 min. The pellet was dissolved in 1.5 ml of chloroform/methanol (2:1), centrifuged again, and the supernatant was submitted to chromatography.

2.2. Chromatography on CM cellulose (method A)

For 30 g cells as starting material, 6 g Servacel CM 32-cellulose were washed successively with 0.5 M NaOH, water, 0.5 M HCl, water, glacial acetic acid, water, 25% ammonia, and water, according to [8]. A glass column (d = 0.8 cm) was filled up to 6.5 cm with the material, and then washed with approximately 6 ml chloroform/methanol (2:1) The CM-cellulose column was loaded with the proteolipid, then washed with 4.2 ml chloroform/methanol (2:1), 8.4 ml chloroform/methanol (1:1), and 4.2 ml chloroform/methanol/water (5:5:1). The c-subunit was eluted with an ammonium acetate gradient run in the chloroform/methanol/water (5:5:1) solvent. Beginning at 5 mM ammonium acetate, the column was washed with 8.4 ml of each concentration up to 25 mM ammonium acetate in steps of 5 mM. The fractions were analyzed for absorption at 280 nm, and pooled fractions were submitted to SDS-gel electrophoresis (see below). Isolated c-subunit was stored at −22°C.

2.3. Anion-exchange HPLC (method B)

Subunit c was separated from contaminants in the organic solvent phase on a 4.6 mm × 10 cm WAX 300 anion-exchange HPLC column (Synchropak®, Hewlett Packard). The method is a slight modification of that described [10]. A 50–200 μl sample of the supernatant (0.23–1 mg protein) or 100 ml of the pooled fractions from CM-cellulose chromatography (22 μg protein) was applied to the column which was subsequently washed with 5 ml of chloroform/methanol/water (4:4:1) at 1 ml/min. A linear gradient of chloroform/methanol/water (4:4:1) to 60% chloroform/methanol/0.9 M aqueous ammonium acetate (4:4:1) was run over a period of 25 min at 1 ml/min, followed by reequilibration with chloroform/methanol/water (4:4:1) for 20 min. Protein eluting fom the column was detected by absorption at 280 nm (Fig. 1). Samples were analyzed by SDS-gel electrophoresis and stored at −22°C. Subunit c was shown to elute at 19.3 min (14.3 min after starting the gradient).

2.4. Reaction with [^{14}C]DCCD and determination of the modification

Labeling of the c-subunit was performed essentially as described [11]. 13 μl of 2% dodecyl-β-D-maltoside in H_2O were added to 29 μg of the c-subunit, dissolved in chloroform/methanol, and the organic solvents were blown off with a gentle stream of N_2. 0.117 ml of the incubation mixture containing buffer composed as indicated in the figure legends and 40 μM [^{14}C]DCCD (54 μCi/μmol) were added to the detergent-solubilized c-subunit, giving a final volume of 0.13 ml, and incubated at 25°C. After different time intervals, 28 μl samples were added to 252 μl ice-cold trichloroacetic acid (final concentration 10%). After centrifugation, the pellet was dried in a speed-vac, and applied to SDS-PAGE as described below. The gels were washed with 10% acetic acid for 50 min, placed on Whatman 3MM, dried under vacuum, placed onto phosphor screen in a storage cassette, and exposed for 6–12 days. Radioactive bands were detected and areas quantified with a PhosphorImager (Molecular Dynamics). As standards, [^{14}C]DCCD probes were dissolved in sample buffer, applied to SDS-PAGE, and exposed together with the protein gels. The radioactivity of the standards (100–4500 dpm) were quantified with parallel samples by β-scintillation counting. Radioactivity volumes observed with the PhosphorImager could thus be converted into dpm, and with the specific radioactivity of the [^{14}C]DCCD (54 nCi/nmol) the amount of [^{14}C]DCCD present on the gel could be converted into nmol.

2.5. Rate of labeling of the c-subunit with [^{14}C]DCCD

The rates of subunit c labeling by [^{14}C]DCCD were obtained from the increase of radioactivity volumes with incubation time and converted into nmol DCCD bound × min^{-1} × nmol c-subunit^{-1}. Second order rate constants were calculated from these values by division through the DCCD concentration (40 μM under our conditions).

2.6. Polyacrylamide gel electrophoresis

The method used for polyacrylamide gel electrophoresis in the presence of sodium dodecyl-sulfate has been described [12]. Samples in organic solvents were dried in a speed vac, the pellet was dissolved in 10–20 ml of sample buffer, and diluted 1:2 in H_2O. All samples were incubated for 5 min at 100°C in a water bath before application to the gel. Silver staining was performed as described [13].

2.7. Protein assays

Protein was determined by the enhanced protocol of the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA) using bovine serum albumin (50–250 μg/ml) as standard. Samples containing organic solvents were dried in a speed vac and resolved in 10 μl of 2% dodecyl-β-D-maltoside. 90 μl water was added and assays were performed as described in the protocol.

3. Results

3.1. Purification of subunit c of the P. modestum *ATPase*

Subunit c was extracted from the particulate cell fraction with chloroform/methanol, and precipitated with diethylether. After this purification a band corresponding to subunit c was clearly visible after SDS-PAGE (Fig. 1) but contaminating proteins of higher molecular weight were still present. Most of these contaminants could be removed by chromatography on CM-cellulose or by anion-exchange HPLC. After combining the two columns, pure subunit c was obtained as shown by SDS-PAGE (Fig. 1). The diffuse stain in the upper part of the gel was also seen in the control (loading buffer without protein) and only by silver staining, and therefore certainly does not derive from contaminating protein(s).

3.2. Labeling of the isolated subunit c by [^{14}C]DCCD

The specific reaction of Glu-65 of subunit c of the

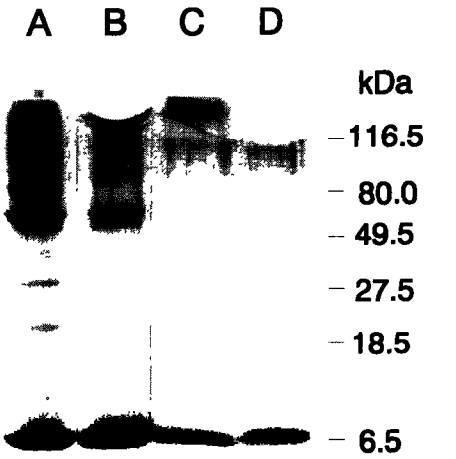

Fig. 1. Silver stained SDS-PAGE of subunit c from *P. modestum* at different purification steps. (A) Particulate cell fraction extracted with chloroform/methanol (5.8 μg protein), (B) subunit c purified by anion exchange HPLC (5.1 μg protein), (C) subunit c purified by CM-cellulose chromatography (2.5 μg protein), (D) subunit c purified by CM-cellulose chromatography and subsequent anion exchange HPLC (10.0 μg protein). Also shown are the migrations of molecular weight markers.

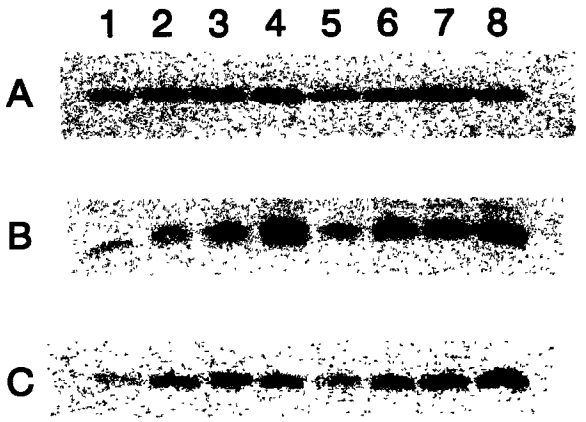

Fig. 2. Kinetics of labeling of the purified c-subunit of the *P. modestum* ATPase with [^{14}C]DCCD at different pH values. Detergent-solubilized c-subunit was incubated with 40 μM [^{14}C]DCCD (54 μCi/μmol) at pH values of 5.5, 6.1, 7.5, or 9.0. The endogenous Na^+ concentration was about 40 μM (lanes 5–8); 20 mM NaCl had been added to the experiments recorded in lanes 1–4. Samples were taken after 5 min (lanes 1 and 5), after 10 min (lanes 2 and 6), after 25 min (lanes 3 and 7), and after 40 min (lanes 5 and 8), precipitated with trichloroacetic acid, and applied to SDS-PAGE as described in section 2. (A) Labeling incubation mixture at pH 5.5. The grey scale goes linearly from 15 counts (background) to 59 counts after 8 days exposure. (B) Labeling incubation mixture at pH 6.1. The grey scale goes linearly from 27 counts (background) to 125 counts after 10 days exposure. (C) Labeling incubation mixture at pH 7.5. The grey scale goes linearly from 18 counts (background) to 45 counts after 12 days exposure. Under the same conditions, no labeling at all could be recorded at pH 9.0.

ATPase with DCCD was apparently retained in subunit c isolated in chloroform/methanol because [^{14}C]DCCD became incorporated into the protein upon exposure in the organic solvent. To study the effect of pH and Na^+ concentration on DCCD labeling, subunit c was transferred into an aqueous dodecyl-β-D-maltoside-containing solution. Incubation of this material with [^{14}C]DCCD resulted in an incorporation of the radioactive label into subunit c as shown by SDS-PAGE and subsequent autoradiography.

3.3. *Effect of H^+, Na^+, Li^+, K^+, or EIPA on the labeling with [^{14}C]DCCD*

The effect of pH and Na^+ concentration on the labeling of the c-subunit with [^{14}C]DCCD is shown in Fig. 2. It is evident from these results that 20 mM Na^+ caused partial protection from the modification reaction at pH 6.1 or pH 7.5 but not at pH 5.5. Quantitative evaluation of the data revealed a linear increase of the radioactivity incorporated with time from which the rate constants could be calculated. Secondary order rate constants were about 70 $min^{-1} \cdot M^{-1}$ at pH 5.5 in the absence as well as in the presence of 20 mM Na^+ or at pH 6.1 in the absence of Na^+. At the latter pH value the rate constant decreased to 50 $min^{-1} \cdot M^{-1}$ upon Na^+ addition (20 mM). At pH 7.5 the rate constants were 20 or 10 $min^{-1} \cdot M^{-1}$ in the absence or presence of 20 mM Na^+, respectively. No bands at all were observed on incubation at pH 9.0 after 12 days of exposure. Please note, that for getting the best contrast for each series (Fig. 2A–C) different background levels, maximal count levels, and exposure times had to be chosen (see legend to Fig. 2).

In summary, these results indicate an enhanced reactivity of subunit c with DCCD at acidic pH values and partial protection from this modification by Na^+ ions at pH 6.1 or, more pronounced, at pH 7.5. At pH 6.1, 150 mM LiCl had a similar protecting effect on DCCD labeling of subunit c as 20 mM NaCl, but 150 mM KCl was without effect. Interestingly, preincubation of the c-subunit with 1.5 mM EIPA severely reduced the rate of labeling with [^{14}C]DCCD to about 20% of the control after 40 min incubation. EIPA thus obviously interferes with the DCCD-reactive site of subunit c.

4. Discussion

Subunit c of F_1F_o-ATPases contains a conserved acidic amino acid residue in the middle of the C-terminal membrane spanning α-helix which is crucial for the translocation of protons (or Na^+ ions in the case of the *P. modestum* enzyme) [6]. The highly specific modification of this residue by DCCD abolishes H^+ or Na^+ translocation through F_o and coupled ATP synthesis or hydrolysis.

In a recent study with the *P. modestum* ATPase we observed an enormously high rate of the modification reaction at acidic pH ($k_2 = 3 \times 10^5$ $min^{-1} \cdot M^{-1}$ at pH 6.0) which decreased at increasing pH values following a titration curve with pK = 7.0 [8]. Also important was a specific protection by Na^+ concomitant with a pK shift into the more acidic range. It was concluded from these and other results that Na^+ and H^+ compete for binding to the reactive Glu-65 residue of subunit c, and that DCCD reacts with Glu-65 in its protonated form whereas Na^+ binds to the deprotonated residue. This binding of Na^+ caused activation of ATPase activity and is regarded as an essential step in the translocation of the alkali ion.

We have now isolated subunit c from the *P. modestum* ATPase to investigate whether the isolated c-subunit exhibited similar properties as the ATPase complex with respect to modification by DCCD. Our results indicated that DCCD indeed reacted with isolated subunit c. Analogous observations have been reported for the isolated c-subunit of the *E. coli* enzyme [7]. The reaction of the c-subunit from *P. modestum* was dependent on pH which is similar to the reaction of DCCD with the ATPase complex. About equal rates were found at pH 5.5 or 6.1. At pH 7.5 the rate dropped to about one third and at pH 9.0 the rate of labeling was beyond the sensitivity of the assay. The second order rate constant at pH 6.1 and 25°C was about 0.7×10^2 $min^{-1} \cdot M^{-1}$. Taking into ac-

count the stoichiometry of approximately 10 c-subunits in the ATPase complex the comparable rate constant would be 3×10^4 $min^{-1} \cdot M^{-1}$. Isolated subunit c thus reacts about 400 times slower with DCCD than the c-subunit does within the F_1F_o complex. The enhanced reactivity of the F_1F_o complex with DCCD may result from assembly of several c-subunits or from the interaction of c-subunits with other F_o or F_1 subunits. SDS-PAGE of the purified *P. modestum* ATPase indicated a tight complex of about 6 c-subunits that even resisted boiling in SDS for 3 min [1]. The c-subunit isolated by extraction with chloroform/methanol, however, exclusively existed as monomer. The strong positive cooperativity of at least three Na^+-binding sites on the enzyme at pH 9.0 is also in accord with an interaction of several c-subunits in the ATPase complex [5]. This cooperativity may be at least in part responsible for the observed highly efficient protection of c-subunits of F_1F_o from DCCD labeling in the presence of Na^+ ions. With isolated c-subunits, Na^+ ions also produced some protection from labeling with DCCD, most markedly at pH 7.5 where the rate decreased to about one half in presence of 20 mM NaCl. We conclude therefore that in isolated subunit c the Na^+ binding site at the DCCD-reactive amino acid residue (Glu-65) is partially retained. We are unable to decide whether the more pronounced protection of subunit c labeling by Na^+ in the complex may result from a more perfect Na^+ binding site provided by the oligomeric stucture of c-subunits or from Na^+-binding ligands provided by subunit a or, less likely, by subunit b.

In our previous paper we demonstrated protection from inactivation of the ATPase by DCCD in the presence of EIPA which was taken as evidence that EIPA and DCCD bind at the same site [5]. This conclusion is clearly confirmed by our present results which show strong protection from labeling of isolated subunit c with DCCD in the presence of EIPA. Therefore a guanidino group-like structure present in EIPA or the transition state derived from the reaction of DCCD with the proton from Glu-65 may be essential for the binding at the active site of subunit c, and may mimic binding of the guanidino group of an arginine side chain that is essential for catalysis as has been proposed previously.

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References

[1] Laubinger, W. and Dimroth, P. (1988) Biochemistry 27, 7531–7537.
[2] Laubinger, W., Deckers-Hebestreit, G., Altendorf, K. and Dimroth, P. (1990) Biochemistry 29, 5458–5463.
[3] Kaim, G., Ludwig, W., Dimroth, P. and Schleifer, K.H. (1992) Eur. J. Biochem. 207, 463–470.
[4] Kluge, C. and Dimroth, P. (1992) Biochemistry 31, 12665–12672.
[5] Kluge, C. and Dimroth, P. (1993) Biochemistry 32, 10378–10386.
[6] Fillingame, R.H. (1990) The Bacteria 12, 345–391.
[7] Girvin, M.E. and Fillingame, R.H. (1993) Biochemistry 32, 12167–12177.
[8] Fillingame, R.H. (1976) J. Biol. Chem. 251, 6630–6637.
[9] Graf, T. and Sebald, W. (1978) FEBS Lett. 94, 218–222.
[10] Hermolin, J. and Fillingame, R.H. (1989) J. Biol. Chem. 264, 3896–3903.
[11] Uchida, E., Ohsumi, Y. and Anraku, Y. (1985) J. Biol. Chem. 260, 1090–1095.
[12] Kluge, C. and Dimroth, P. (1993) J. Biol. Chem. 268, 14557–14560.
[13] Wray, W., Boulikas, T., Wray, V.P. and Hancock, R. (1981) Anal. Biochem. 118, 197–203.